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Immunotherapy has been shown to be effective adjuvant in the management of septic shock. A neuropenic rat model of septic shock induced by infection with pseudomonas aeruginosa 12.4.4 was used to determine the relative efficacy of single, double, and triple combination immunotherapy. A Pseudomonas O serotype-specific, opsonophagocytic monoclonal antibody, polyclonal J5 antiserum and a Mab sirected against tumor necrosis factor (TNF) were studied as single therapy and in combination of all three immunotherapeutic agents resulted in a 77% survival rate. This level of of protection was superior to that acheived with any combination of two antibody treatments or single antibody therapy or compared wtth the control group. Immunotherapy directed against multiple steps of the septic process is more activ e than single or double antibody regimens and may offer an improved approach to the adjunctive treatment of septic shock.

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The Efficacy of Combination Immunotherapy in Experimental *Pseudomonas* Sepsis

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Immunotherapy has been shown to be an effective adjuvant in the management of septic shock. A neutropenic rat model of septic shock induced by infection with *Pseudomonas aeruginosa* 12.4.4 (Fisher immunotype 6) was used to determine the relative efficacy of single, double, and triple combination immunotherapy. A *Pseudomonas* O serotype–specific, opsonophagocytic monoclonal antibody (MAb), polyclonal J5 antiserum, and a MAb directed against tumor necrosis factor-α (TNF) were studied as single therapy and in combination. The combination of all three immunotherapeutic agents resulted in a 77% survival rate (33/43 animals). This level of protection was superior to that achieved with any combination of two antibody treatments (50%–60% survival; $P = .029$) or single antibody therapy (25%–43% survival; $P < .001$) or compared with a control group (0/25 survivors; $P < .0001$). Immunotherapy directed against multiple steps of the septic process is more active than single or double antibody regimens and may offer an improved approach to the adjunctive treatment of septic shock.

Despite the availability of potent antimicrobial agents that are active against a broad spectrum of gram-negative bacteria, the mortality from sepsis caused by these organisms has changed little in the past decade [1–3]. Consequently, there has been considerable interest in immunotherapeutic measures to supplement conventional treatment with antimicrobial agents and supportive care.

Since there is a wide spectrum of serotypes among clinical isolates retrieved from the blood, the preparation of serotype-specific antibody therapy has been considered impractical. Instead, efforts have been directed toward identifying epitopes in the lipopolysaccharide (LPS) core region of Enterobacteriaceae and *Pseudomonas aeruginosa* that might be widely shared. Antibodies prepared against epitopes on a deep rough (Re chemotype) LPS mutant of *Salmonella minnesota* and against epitopes on a rough (Rc) mutant (J5) of *Escherichia coli* O111:B4 have been shown to protect against lethal infection with heterologous bacteria in animal models of infection [4–6] and upon direct endotoxic challenge [7, 8]. Clinical studies with human antisera elicited by immunization with a J5 vaccine showed significant protection when given as treatment [9] or prophylaxis [10] of sepsis; however, since the protection was not correlated with the antibody titers to J5 epitope(s), the basis for the improved survival was not determined. Even with a significant reduction in mortality with this adjunctive immunotherapy, however, the mortality exceeded 30% [9].

In assessing possible reasons for this still unacceptably high mortality after therapy with J5 antiserum, we considered that this treatment, designed to neutralize the toxic properties of LPS, might not be efficacious at all stages of the septic process. For example, once LPS has stimulated macrophages to produce cytokines, such as tumor necrosis factor-α (TNF) or interleukin-6 (IL-6), an antibody to LPS would not be expected to modulate the ensuing cytokine cascade. Similarly, endotoxin-neutralizing antibodies might not facilitate the opsonic clearance of invading bacteria by phagocytes before these bacteria achieve concentrations in the blood sufficient to initiate a cytokine response. We have previously demonstrated in an animal model of sepsis the protective efficacy of a monoclonal antibody (MAb) to TNF both alone and in combination with antibiotics or in combination with an LPS serotype-specific MAb [11, 12]. Since it is impossible to determine at the bedside at which stage of sepsis an individual patient might be, we hypothesized that a combination of antibodies, each directed at a different stage of the septic process, might be more efficacious in improving survival than an antibody directed at any one step.

Materials and Methods

Challenge organism. The challenge organism used in these experiments was a smooth LPS-bearing, serum-resistant, human blood isolate of *P. aeruginosa* (12.4.4, originally provided

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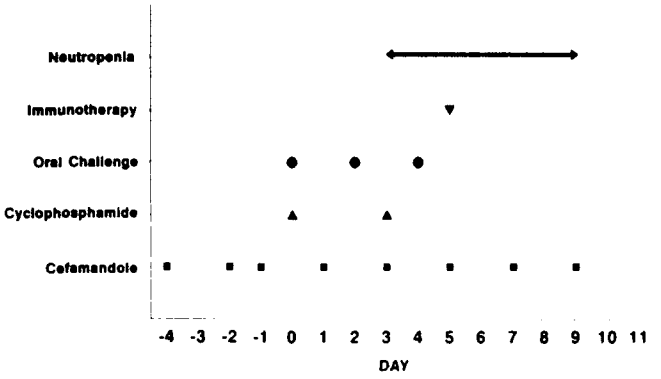

Figure 1. Protocol for neutropenic rat model. Immunotherapeutic regimens were given as single intravenous dose at onset of fever during period of neutropenia.

by A. McManus, United States Army Institute of Surgical Research, San Antonio, TX). The organism belongs to Fisher-Devlin-Gnabasik immunotype 6. The organism was stored in 10% glycerin at −70°C until ready for use. The day before oral challenge, the isolate was incubated overnight in trypticase soy broth (TSB; Becton Dickinson, Cockeysville, MD) at 37°C. The next day, bacteria were suspended in normal saline and adjusted spectrophotometrically to an inoculum size of 10^6 cfu/mL, a dose that resulted in a 90% mortality in previous studies [11, 12].

Animal model. Female albino, pathogen-free, Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 125–150 g were maintained in filtered, biologic safety cages and allowed to eat and drink ad libitum. Intramuscular cefamandole (Eli Lilly, Indianapolis) was administered to each animal at a dose of 100 mg/kg on an every-other-day schedule. Animals were rendered neutropenic by the intraperitoneal administration of cyclophosphamide (Bristol-Myers, Evansville, IN) at a dose of 100 mg/kg at time 0 followed by a second dose of 50 mg/kg 72 h later.

The oral challenge with *P. aeruginosa* 12.4.4 was administered at a dose of 1 mL (10^6 cfu) on days 0, 2, and 4 via an orogastric tube prepared from polyethylene tubing that was free of tissue reaction (Intramedic PE, 160; Clay Adams Division, Becton Dickinson, Parsippany, NJ). All manipulations were done under light CO_2 anesthesia to minimize trauma to the animals. A detailed description of this animal model has been presented previously [11]. Before onset of neutropenia, a patch of fur of ~5 × 5 cm was shaved off to allow for accurate and repeated body temperature recordings. A Horiba noncontact digital infrared thermometer (Markson Science, Phoenix) was used to monitor the animals' body temperature several times daily. At the onset of fever (defined as a recorded temperature >38.0°C), antibody treatment was administered intravenously as a single bolus injection. No antimicrobial agents active against *P. aeruginosa* were administered to the animals. The animals were observed daily for 12 days after the initial dose of cyclophosphamide. Any animal that died during the experimental period was subjected to necropsy within 24 h. The fundamental elements of the animal model are depicted in figure 1.

Antibody treatments. A murine-derived MAb directed against the O-specific side chain of *P. aeruginosa* 12.4.4 (anti-O MAb) was prepared as previously described [13]. This MAb, designated 11.4.1, is of the IgG1 isotype and was given intravenously at a dose of 2.5 mg/kg. The antibody has been previously shown to possess serotype-specific opsonophagocytic activity and, at this dose, to protect rodents from lethal challenge with *P. aeruginosa* 12.4.4. This antibody lacks measurable anti-endotoxin activity and fails to bind to the core glycolipid structure of *P. aeruginosa* LPS on Western blot [12].

A hamster-derived anti-murine TNFα MAb (TN3 19.12, anti-TNF MAb) was originally provided by R. Schreiber (Washington University, St. Louis). This is an IgG MAb that neutralizes natural rat TNFα as well as murine TNF in an L929 cytotoxicity assay at a level of 19 ng/unit of TNF [14]. This MAb was prepared for these studies by Celltech and was given at 20 mg/kg intravenously, a dose that afforded 44% protection in a previous study [12].

A polyclonal antiserum directed against the core glycolipid of bacterial endotoxin was prepared by immunization of New Zealand White rabbits with a heat-inactivated vaccine of *E. coli* J5 by previously described methods [8]. The J5 antiserum was given at a dose of 1.5 mL/kg based on the expected ED_{50} derived from preliminary experiments (data not shown).

Animals in the control group received an irrelevant MAb (MAb L2 3D9) that is hamster derived and directed against recombinant murine IL-2. This MAb does not react with natural mouse or rat IL-2. It was administered intravenously at a dose of 20 mg/kg. Since MAbs, even irrelevant ones, may have a deleterious effect in the animal model, an additional control, preimmune rabbit serum, was also administered to a total of 14 animals in the control group.

Blood determinations and necropsy studies. Blood samples were obtained from the retroorbital plexus from each animal 24 h before the first dose of cyclophosphamide, at the onset of fever, and 24 h after the intravenous administration of immunotherapy. Complete blood counts were done to ensure that neutropenia had been achieved. Serum TNF levels were determined by the L929 fibroblast cytotoxicity assay as previously described [15]. Blood cultures were done by the addition of 1.5 mL of TSB to 0.5 mL of aseptically obtained blood specimens and incubation for 72 h at 37°C.

At necropsy, tissue from heart, lung, spleen, liver, and jejunum was cultured for each animal. Non–lactose-fermenting, oxidase-positive colonies that appeared on MacConkey's agar were further identified by agglutination reactions with a polyvalent *P. aeruginosa* antisera set (Difco, Detroit) to ensure that the challenge strain was immunotype 6. Histologic sections of lung, cecum, and renal tissue were obtained from 5 lethally infected animals in the control group.

Data analysis. Survival analysis between multiple treatment groups was compared by the Kruskal-Wallis one-way analysis of variance statistic [16]. The survival analysis was based on an intention-to-treat with the inclusion of all animals given cyclophosphamide. No animals were excluded from the analysis. Serum TNF levels were compared by analysis of variance or two-sample *t* test where appropriate. Frequency comparisons

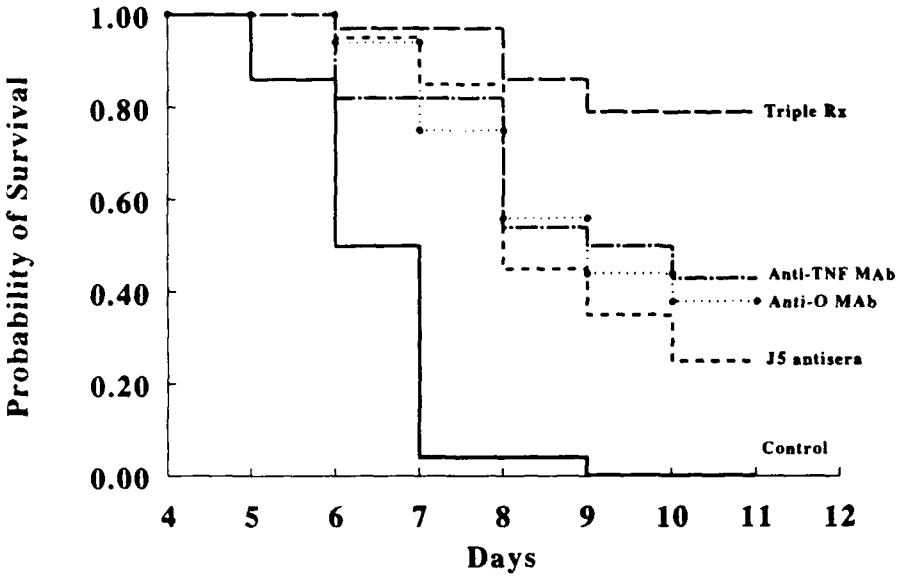

Figure 2. Survival rate of treatment groups receiving single antibody treatments compared with combination of all three antibody treatments and control group. Control = irrelevant monoclonal antibody (MAb) L2 3D9 (n = 11) and normal rabbit serum (n = 14) (total, 25); anti-TNF MAb = anti-tumor necrosis factor MAb TN3 19.12 (n = 28); anti-O MAb = serotype-specific, anti-*Pseudomonas aeruginosa* 12.4.4 MAb, MAb 11.14.1 (n = 16); J5 antiserum = polyclonal rabbit anti-core glycolipid antibody (n = 20); triple Rx = combination treatments (n = 43). Data for treatment groups were combined from two separate experiments. Data for control groups were combined from four separate experiments.

were made by the χ^2 analysis-of-contingency table method. All analyses were two-sided, and $P < .05$ was considered significant. Results are expressed as mean ± SE.

Results

Outcome in animals receiving single immunotherapy. Each antibody treatment significantly improved the survival rate compared with that of the control group ($P < .005$; figure 2). The animals in the control group uniformly succumbed to multisystem infection with *P. aeruginosa* 12.4.4. Since this result occurred with either irrelevant MAb (n = 11) or normal rabbit serum (n = 14), we analyzed both groups as one control group. All lethally infected animals had a culture positive for the challenge strain of *P. aeruginosa* from the cecum as well as at least one other infected organ. Most animals (85%) had positive cultures in all organs. Necropsy specimens consistently revealed the challenge strain in multiple organs, and histologic examination found evidence of acute tubular necrosis and pulmonary vascular congestion with interstitial edema.

Outcome in animals receiving double or triple combination immunotherapy. Animals receiving a combination of any two of the antibody treatments had a significantly improved survival rate compared with groups receiving any single antibody therapy (figures 2, 3; $P < .05$). The simultaneous administration of all three antibodies—anti-TNF MAb, anti-core glycolipid polyclonal antiserum, and anti-O MAb—consistently provided the best protection in this neutropenic rat model, with survival rates of 77% (33/43). This result is highly significant compared with treatment outcomes from single antibody therapy (figure 2; $P < .001$ for each comparison). The regimen combining all three antibodies also achieved a survival benefit significantly greater than survival rates from the double antibody treatments (figure 3; $P < .029$ for each comparison).

Culture determinations and cytokine levels. Frequency of bacteremia with the challenge strain, *P. aeruginosa* 12.4.4, was 93% at the onset of fever during the period of neutropenia. None of the blood cultures obtained in the pretreatment phase revealed *P. aeruginosa*. The serotype-specific anti-O MAb–treated animals did have a significantly lower frequency of bacteremia at 24 h after immunotherapy (27%) compared with control (51%) or treatment groups that did not receive anti-O MAb (63%) ($P < .01$). The difference in frequency of bacteremia in animals receiving either the anti-TNF MAb or anti-J5 serum compared with the control group did not attain statistical significance. All animals that died were subjected to autopsy within 12 h. The challenge strain was routinely cultured from liver, spleen, lung, and cecum. At the end of each study, surviving animals were sacrificed and their organs cultured. Liver, spleen, and lung cultures were routinely sterile.

Pretreatment serum TNF levels were 18.0 ± 16.8 pg/mL and did not differ in any treatment group. However, at the onset of fever during the period of neutropenia, the mean serum TNF levels were markedly elevated, 630 ± 105 pg/mL ($P < .001$). Administration of the anti-TNF MAb resulted in a precipitous decline in TNF levels by 24 h to 30.6 ± 12.6 pg/mL (table 1). This result was significantly different from that seen in the control group, in which the mean TNF level remained elevated at 488.0 ± 89.4 pg/mL ($P < .0001$). The J5 antiserum–treated animals also experienced a measurable decline in TNF levels 24 h after immunotherapy to 58.8 ± 12.9 pg/mL. This level was significantly lower than that in the control group ($P < .001$). In contrast, anti-O MAb treatment did not result in a significant diminution of the serum TNF levels compared with controls (433.0 ± 34.6 vs. 480.0 ± 89.4 pg/mL; P = not significant).

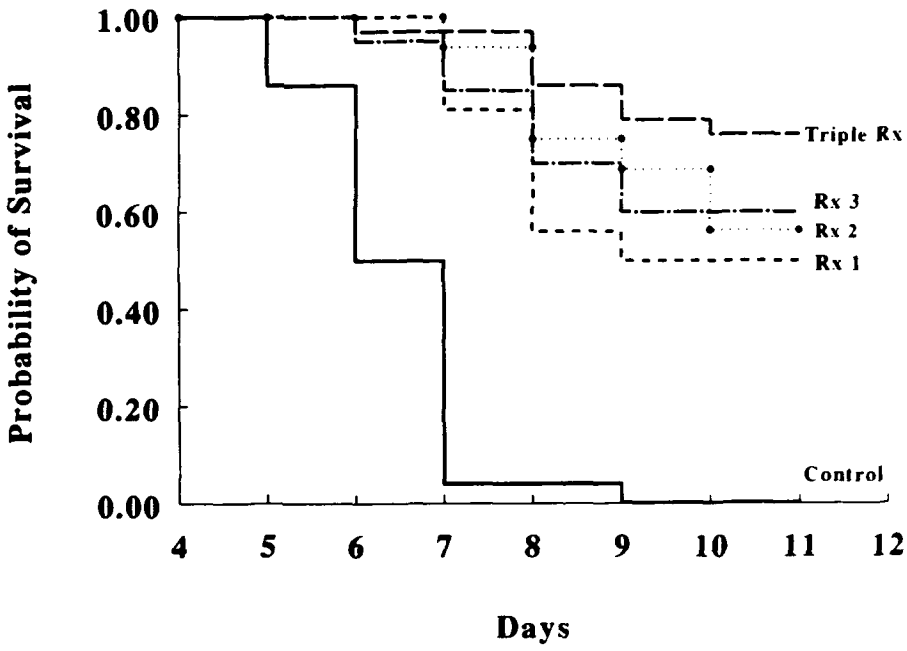

Figure 3. Efficacy of three different double immunotherapeutic regimens compared with triple immunotherapy and control group. Control = irrelevant monoclonal antibody (MAb) L2 3D9 (n = 11) and normal rabbit serum (n = 14) (total, 25); Rx 1 = polyclonal J5 antiserum against core glycolipid of *Escherichia coli* J5 lipopolysaccharide + serotype-specific, anti-*Pseudomonas aeruginosa* 12.4.4 MAb 11.14.1 (n = 16); Rx 2 = anti-tumor necrosis factor MAb TN3 19.12 + MAb 11.14.1 (n = 16); Rx 3 = TN3 19.12 + polyclonal anti-core glycolipid antiserum (n = 17); triple Rx = combination of all three antibody treatments (n = 43). Identical control and triple immunotherapy data used in figure 2 were used for this figure. Double immunotherapy data (Rx 1–3) were combined from two experiments.

Discussion

These studies demonstrate that a combination of antibodies is more effective in protecting against lethal bacterial infection than any one of the antibodies given either individually or in combination with a second preparation (i.e., double combination immunotherapy). Thus, these data support the concept of a multistep septic process (figure 4). Gram-negative bacteria that colonize the mucocutaneous system of a host can invade the bloodstream directly, or, as in the case of *Pseudomonas* burn wound sepsis, can elaborate endotoxin that circulates in the blood. In the former instance, host defenses may limit the dissemination of the bacteria from the site of entry: Complement-mediated bacteriolysis and neutrophil-mediated, antibody-dependent opsonophagocytic killing are often able to kill the potential pathogens. When this occurs, endotoxins may be transiently liberated and cause a septic clinical appearance. Similarly, antibiotics may also liberate endotoxins in the course of their bactericidal activity [17].

The endotoxin on bacterial pathogens that can evade these host defenses, or free endotoxin liberated from killed bacteria or absorbed from wounds such as burns, may then interact with target cells of the host, primarily macrophages and probably endothelial cells. The mode of interaction be-

Table 1. Tumor necrosis factor (TNF) levels after immunotherapy in a rat model of *Pseudomonas* sepsis.

Treatment group (n)	Serum TNF (pg/mL) 24 h after therapy	P*
Control (25)	488.0 ± 89.4	
Anti-TNF MAb (28)	[illegible] ± 12.6	<.0001
J5 antiserum (20)	58.8 ± 12.9	<.001
Anti-O MAb (16)	433.0 ± 34.6	NS

NOTE. MAb, monoclonal antibody; NS, not significant.
* Compared with control group by analysis of variance.

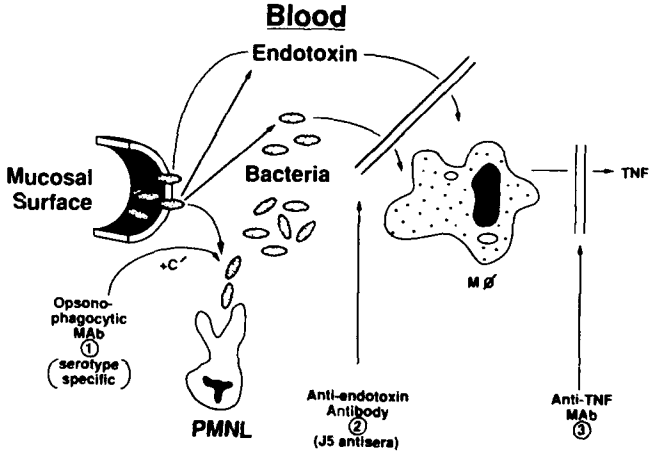

Figure 4. Proposed model of gram-negative bacillary sepsis as multistep process. Bacteria from mucosal surface or gut lumen translocate into blood compartment. Bacteria may encounter neutrophils (PMNL), which may ingest bacteria in conjunction with complement (C'). Lipopolysaccharide from these bacteria may encounter macrophages (Mϕ), which may then elaborate cytokines, such as tumor necrosis factor-α (TNF). Antibody treatments may modulate at each step. J5 antisera = polyclonal lapine antisera raised by J5 *Escherichia coli* vaccine. MAb = monoclonal antibody.

tween endotoxin and cell is under intense scrutiny and may occur via direct LPS receptors, indirectly via "docking" proteins in the serum (e.g., LPS-binding protein) that bind to cellular receptors such as CD14, or through a combination of these receptors [18–20].

Once the LPS has interacted with the cell, a cytokine cascade is initiated. Such cytokines may serve a beneficial role in host defenses when part of a highly regulated and orchestrated host response. In the case of sepsis, however, this cytokine cascade may become dysregulated such that positive feedback loops are initiated and deleteriously high levels of cytokines previously associated with sepsis, such as TNF and IL-6, become generated [15].

Such a paradigm of sepsis suggests that the process would be amenable to immunomodulation at several distinct points. Antibody directed toward specific bacterial surface determinants, such as capsular polysaccharide or O serogroups of endotoxin, may enhance opsonophagocytic clearance of the bacteria such that a continually increasing source of endotoxin (proliferating bacteria) does not progress to a level sufficient to initiate cytokine production. Indeed, recent data suggest that there may be a quantitative relationship between the number of intact bacteria that interact with macrophages and their ability to generate TNF in vitro. More than 10^4 cfu of *E. coli* were required to induce detectable TNF production by cells of the murine monocyte/macrophage cell line RAW 264.7 [21]. Opsonophagocytic antibody might also promote the clearance of non-LPS bacterial virulence factors, such as elastase and exotoxin A.

Second, antibody directed toward the endotoxin core may neutralize the biologic effects of endotoxin before its interaction with target cells. Finally, antibody that neutralizes the TNF generated by the interaction of LPS with the target cell may lessen the severity of the process and limit the cytokine cascade [22]. Encouraging results have also been obtained in the treatment of sepsis with another modulator of the cytokine response in sepsis, IL-1 receptor antagonist [23]. Clinical studies in humans have been done or are in progress with antibodies directed toward each of these stages of sepsis [9, 24, 25].

We speculate that the 30% mortality among those patients receiving J5 antiserum was among individuals who either received an inadequate dose or among patients who were in early "pre-endotoxin" or in late "post-macrophage" stages of sepsis (figure 4). Endotoxin-neutralizing antibody might not be expected to intercede at these stages of sepsis. Since at the bedside it is impossible to know at which stage of sepsis a patient might be, a combination of interventions might be more highly protective, as was demonstrated in these animal studies.

This neutropenic rat model closely mimics a relatively common clinical situation: Patients rendered neutropenic from cytotoxic chemotherapy often receive concomitant antimicrobial agents. The latter intervention often overcomes colonization resistance to opportunistic pathogens such as *P. aeruginosa* and enables these bacteria to colonize the gut, from where they may later invade and disseminate within the host. As is the case in the patient, the exact stage of bacterial infection cannot be determined, but the level of bacterial inoculum necessary to initiate an infection is physiologic: Animals are lethally infected via the oral route with an inoculum of *Pseudomonas* organisms usually found on one tomato [26].

This study demonstrates that anti-TNF Mab given as treatment 3–5 days after initial bacterial challenge and after the onset of a systemic response to the infection (fever) does significantly protect against lethal infection and decrease circulating TNF levels. In contrast, previous studies using either mouse or baboon models of sepsis required the anti-TNF Mab to be given as prophylaxis before the LPS [27] or live bacterial challenge [27, 28] for the protective effect of the Mab to be evident. The differences between those studies and our results are most readily explained by the differences in the animal models of sepsis.

The neutropenic rat is a model of infection in which sepsis is achieved through the ability of bacteria to evade host defense mechanisms by surface or extracellular virulence determinants. Bacteria that lack these virulence determinants are efficiently cleared by the host and are unable to establish an infection. In contrast, for such avirulent bacteria to elicit a cytokine response, it is necessary to administer an inoculum sufficiently large to overwhelm or intoxicate the host defense system. Bolus infusion of large doses of avirulent bacteria or LPS differ little in the physiologic effects induced [29]. Both stimuli induce a cytokine response that differs substantially in both peak levels of cytokine achieved and the kinetics of that response from what has been reported during naturally occurring human sepsis [27–30]. There is some evidence from the measurement of TNF levels in septic patients, however, to suggest that the neutropenic rat infection model more accurately mimics both the clinical aspects of sepsis and the physiology of circulating TNF in humans than do intoxication models, such as the baboon sepsis models [31–33]. In the former instance, doses of anti-TNF MAb similar to those used in intoxication models may have a better opportunity to neutralize TNF long after a known bacterial challenge.

It is also noteworthy that in this neutropenic rat model, polyclonal lapine antiserum prepared after immunization with a J5 vaccine provided significant protection upon heterologous challenge compared with that seen with normal rabbit serum. The J5 vaccine was prepared from an isolate originally provided by E. Ziegler (University of California Medical Center, San Diego), and the immunizing regimen was identical to one described earlier [8]. J5 antiserum that previously had been shown to be functionally active against

a heterologous infection in a neutropenic rabbit model and against heterologous LPS in a dermal Shwartzman model was later shown to reduce the mortality from septic shock caused by gram-negative bacteremia in a clinical study [9]. The moiety responsible for the protective effect in the J5 antiserum is not known. Many studies reported since then, however, have been unable to demonstrate functional activity in other animal models [34–36], including the dermal Shwartzman assay [36]. Since none of these latter preparations advanced to clinical study, it is difficult to assess the relevance of the animal models used in these studies to the clinical situation. The rat may be preferable to the lapine model for testing J5 antiserum: The animals are smaller, easier to handle, and less expensive, so larger numbers of animals can be used for statistical analysis of the data.

In summary, these studies support the concept that sepsis is subject to successful immunomodulation at multiple points, even after its onset. The results achieved with triple combination immunotherapy, 77% protection, were obtained in the absence of antibiotic therapy. The addition of antimicrobial agents to the antibody treatment may provide enhanced protection [37]. Finally, this neutropenic model of sepsis may be a feasible model in which to evaluate preparations designed to neutralize the biologic effects of endotoxin.

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